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A13

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> C07K 13/00, C12N 5/00, 15/00 C07H 15/12, 17/00, G01N 33/566 A61K 48/00	<b>A1</b>	<b>(11) International Publication Number:</b> WO 93/23431 <b>(43) International Publication Date:</b> 25 November 1993 (25.11.93)
<b>(21) International Application Number:</b> PCT/US93/04399 <b>(22) International Filing Date:</b> 11 May 1993 (11.05.93)  <b>(30) Priority data:</b> 07/882,771 14 May 1992 (14.05.92) US 07/939,246 2 September 1992 (02.09.92) US  <b>(71) Applicant:</b> BAYLOR COLLEGE OF MEDICINE [US/ US]; One Baylor Plaza, Houston, TX 77030 (US).  <b>(72) Inventors:</b> VEGETO, Elisabetta ; 7510 Brompton #612, Houston, TX 77025 (US). MCDONNELL, Donald, P. ; 10382 Rue Riviere Verte, San Diego, CA 92131 (US). O'MALLEY, Bert, W. ; 639 Ramblewood, Houston, TX 77079 (US). SCHRADER, William, T. ; 2640 Carolina Way, Houston, TX 77005 (US). TSAI, Ming-Jer ; 6014 Charlotte, Houston, TX 77005 (US).		<b>(74) Agents:</b> ADLER, Benjamin, Aaron et al.; Fulbright & Ja- worski, 1301 McKinney, Suite 5100, Houston, TX 77010-3095 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MUTATED STEROID HORMONE RECEPTORS, METHODS FOR THEIR USE AND MOLECULAR SWITCH FOR GENE THERAPY  <b>(57) Abstract</b> <p>The present invention provides mutant proteins of steroid hormone receptors. These mutant proteins are useful in methods of distinguishing a steroid hormone receptor antagonist from a steroid hormone receptor agonist. The present invention also provides plasmids containing mutated steroid hormone receptor proteins and cells transfected with those plasmids. In addition, the present invention provides methods for determining whether a compound is a steroid hormone receptor antagonist or agonist. Also, the present invention provides methods of determining endogenous ligands for steroid hormone receptors. The invention further provides a molecular switch for regulating expression in gene therapy.</p>		

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**MUTATED STEROID HORMONE RECEPTORS,  
METHODS FOR THEIR USE AND  
MOLECULAR SWITCH FOR GENE THERAPY**

5           This invention was supported in part through a grant or award  
from the National Institute of Health.

**BACKGROUND OF THE INVENTION**

1.    Field of the Invention

10           The present invention relates generally to the fields of molecular  
endocrinology and receptor pharmacology. It further relates to molecular  
switches for gene therapy. More specifically, the present invention relates  
to a novel in vivo method for the identification of steroid hormone  
receptor agonists and antagonists and to a molecular switch involving a  
15       modified steroid receptor for up-regulating and down-regulating the  
synthesis of heterologous nucleic acid sequences which have been inserted  
into cells.

2.    Description of the Related Art

20           Steroid receptors are responsible for the regulation of complex  
cellular events, including transcription. The ovarian hormones, estrogen  
and progesterone, are responsible, in part, for the regulation of the  
complex cellular events associated with differentiation, growth and  
functioning of female reproductive tissues. These hormones play also  
important roles in development and progression of malignancies of the  
reproductive endocrine system.

25           The biological activity of steroid hormones is mediated directly by  
a hormone and tissue-specific intracellular receptor. The physiologically  
inactive form of the receptor may exist as an oligomeric complex with

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proteins, such as heat-shock protein (hsp) 90, hsp70 and hsp56. Upon binding its cognate ligand, the receptor changes conformation and dissociates from the inhibitory heteroligomeric complex. Subsequent dimerization allows the receptor to bind to specific DNA sites in the regulatory region of target gene promoters. Following binding of the receptor to DNA, the hormone is responsible for mediating a second function that allows the receptor to interact specifically with the transcription apparatus. Displacement of additional inhibitory proteins and DNA-dependent phosphorylation may constitute the final steps in this activation pathway.

Cloning of several members of the steroid receptor superfamily has facilitated the reconstitution of hormone-dependent transcription in heterologous cell systems. Subsequently, *in vivo* and *in vitro* studies with mutant and chimeric receptors have demonstrated that steroid hormone receptors are modular proteins organized into structurally and functionally defined domains. A well defined 66 amino acid DNA binding domain (DBD) has been identified and studied in detail, using both genetic and biochemical approaches. The ligand (hormone) binding domain (LBD), located in the carboxyl-terminal half of the receptor, consists of about 300 amino acids. It has not been amenable to detailed site-directed mutagenesis, since this domain appears to fold into a complex tertiary structure, creating a specific hydrophobic pocket which surrounds the effector molecule. This feature creates difficulty in distinguishing among amino acid residues that affect the overall structure of this domain from those involved in a direct contact with the ligand. The LBD also contains sequences responsible for receptor dimerization, hsp interactions and one of the two transactivation sequences of the receptor.

Gene replacement therapy requires the ability to control the level of expression of transfected genes from outside the body. Such a "molecular switch" should allow specificity, selectivity, precision safety and rapid clearance. The steroid receptor family of gene regulatory proteins

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is an ideal set of such molecules. These proteins are ligand activated transcription factors whose ligands can range from steroids to retinoids, fatty acids, vitamins, thyroid hormones and other presently unidentified small molecules. These compounds bind to receptors and either up-regulate or down-regulate. The compounds are cleared from the body by existing mechanisms and the compounds are non-toxic.

The efficacy of a ligand is a consequence of its interaction with the receptor. This interaction can involve contacts causing the receptor to become active (agonist) or for the receptor to be inactive (antagonist). The affinity of antagonist activated receptors for DNA is similar to that of agonist-bound receptor. Nevertheless, in the presence of the antagonist, the receptor cannot activate transcription efficiently. Thus, both up and down regulation is possible by this pathway.

The present invention shows that receptors can be modified to allow them to bind various ligands whose structure differs dramatically from the naturally occurring ligands. Small C-terminal alternations in amino acid sequence, including truncation, result in altered affinity and altered function of the ligand. By screening receptor mutants, receptors can be customized to respond to ligands which do not activate the host cells own receptors. Thus regulation of a desired transgene can be achieved using a ligand which will bind to and regulate a customized receptor.

Steroid receptors and other mammalian transcription regulators can function in yeast. This fact, coupled with the ease of genetic manipulation of yeast make it a useful system to study the mechanism of steroid hormone action.

A long felt need and desire in this art would be met by the development of methods to identify steroid hormone receptors agonists and antagonists. The development of such a method will facilitate the identification of novel therapeutic pharmaceuticals. Additionally, the present invention provides a novel approach to regulate transcription in

gene therapy. By using modified steroid receptors and custom ligands, up-regulation and down-regulation of inserted nucleic acid sequences can be achieved.

### SUMMARY OF THE INVENTION

5           An object of the present invention is a modified steroid hormone receptor protein for distinguishing hormone antagonists and agonists.

          An additional object of the present invention is a plasmid containing a modified hormone receptor.

10           A further object of the present invention are transfected cells containing modified hormone receptors.

          Another object of the present invention is a transformed cell containing modified hormone receptors.

          An additional object of the present invention is a method for determining agonist activity of a compound for steroid hormone receptors.

15           A further object of the present invention is a method for determining antagonist activity of a compound for steroid hormone receptors.

          An object of the present invention is a method for determining endogenous ligands for steroid hormone receptors.

20           An object of the present invention is an endogenous ligand for a modified steroid receptor.

          An object of the present invention is a molecular switch for regulated expression of a nucleic acid sequence in gene therapy.

25           An additional object of the present invention is a molecular switch which binds non-natural ligands, anti-hormones and non-native ligands.

          A further object of the present invention is a molecular switch comprised of a modified steroid receptor.

          An additional object of the present invention is a method for regulating expression of nucleic acid sequence in gene therapy.

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A further object of the present invention is a modified progesterone receptor with a native binding domain replaced with GAL-4 DNA.

An additional object of the present invention is to add a more potent activation domain to the receptor.

5 Another object of the present invention is a method of treating senile dementia or Parkinson's disease.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a mutated steroid hormone receptor protein. This mutated steroid hormone receptor protein  
10 is capable of distinguishing a steroid hormone receptor antagonist from a steroid hormone receptor agonist.

In specific embodiments of the present invention, the receptor is selected from a group consisting of estrogen, progesterone, androgen, Vitamin D, COUP-TF, cis-retonic acid, Nurr-1, thyroid hormone,  
15 mineralocorticoid, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , ecdysterone and orphan receptors.

In a preferred embodiment the mutated steroid receptor is mutated by deletion of carboxy terminal amino acids. Deletion usually comprises from one to about 120 amino acids and is most preferably less than about  
20 60 amino acids.

In another embodiment of the present invention, there is provided a plasmid containing a mutated steroid hormone receptor protein. The plasmid of the present invention when transfected into a cell, is useful in determining the relative antagonist or agonist activity of a compound for  
25 a steroid hormone receptor.

In another embodiment of the present invention, there is provided transfected and transformed cells containing a plasmid in which a mutated or steroid hormone receptor protein has been inserted. The transfected cells of the present invention are useful in methods of determining the  
30 activity of a compound for a steroid hormone receptor.

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Another embodiment of the present invention, includes methods of determining whether a compound has activity as an agonist or antagonist as a steroid hormone receptor. These methods comprise contacting the compound of interest with the transfected cells of the present invention and measuring the transcription levels induced by the compound to determine the relative agonist or antagonist activity of the steroid hormone receptors.

In other embodiments of the present invention, there is provided a method of determining an endogenous ligand for a steroid hormone receptor. This method comprises contacting a compound with the transfected cells of the present invention and measuring the transcription levels induced by the compound.

Another embodiment of the present invention is the provision of endogenous ligands for modified steroid hormone receptors that are capable of stimulating transcription in the presence of the transfected cells of the present invention.

A further embodiment of the present invention is a molecular switch for regulating expression of a nucleic acid sequence in gene therapy in humans and animals. It is also useful as a molecular switch in plants and in transgenic animals. The molecular switch is comprised of a modified steroid receptor which includes a natural steroid receptor DNA binding domain attached to a modified ligand binding domain on said receptor.

In specific embodiments of the molecular switch, the native DNA binding domain in unmodified form is used and the ligand binding domain is modified to only bind a compound selected from the group consisting of non-natural ligands, anti-hormones and non-native ligands.

Specific examples of compounds which bind the ligand binding domain include 5- $\alpha$ -pregnane-3,2-dione; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -propinyl-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-



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gonadiene-3-one;  $11\beta$ -(4-acetylphenyl)- $17\beta$ -hydroxy- $17\alpha$ -(1-propenyl)-4,9-estradiene-3-one;  $11\beta$ -(4-dimethylaminophenyl)- $17\beta$ -hydroxy- $17\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one; ( $7\beta$ , $11\beta$ , $17\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2'(3'H)-furan]-3-one; ( $11\beta$ , $14\beta$ , $17\alpha$ )-4',5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one.

In preferred embodiments of the molecular switch, the modified steroid receptor has both the ligand binding domain and DNA binding domain replaced. For example the natural DNA binding domain is replaced with a DNA binding domain selected from the group consisting of GAL-4 DNA, virus DNA binding site, insect DNA binding site and a non-mammalian DNA binding site.

In specific embodiments of the present invention the molecular switch can further include transactivation domains selected from the group consisting of VP-16, TAF-1, TAF-2, TAU-1 and TAU-2.

In a preferred embodiment the molecular switch has a modified progesterone receptor containing a modified ligand binding domain and a GAL-4 DNA binding domain. This molecular switch can also be enhanced by the addition of a TAF-1 or VP16 transactivation domain.

Additional embodiments of the present invention include a method for regulating the expression of a nucleic acid cassette in gene therapy. The method includes the step of attaching the molecular switch to a nucleic acid cassette used in gene therapy. A sufficient dose of the nucleic acid cassette with the attached molecular switch is then be introduced into an animal or human to be treated. The molecular switch can then be up-regulated or down-regulated by dosing the animal or human with a ligand which binds the modified binding site.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention which are given for the purposes of disclosure when taken in conjunction with the accompanying drawings.

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### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the mutagenesis and screening strategy used in the present experiments.

5        Figures 2A, 2B & 2C illustrate the functional and structural characterization of the UP-1 mutant.

Figure 3 shows a western analysis of the mutant human progesterone receptor.

Figure 4 shows the transcriptional activity and hormone binding analysis of wild type and mutant human progesterone receptor constructs.

10       Figures 5A, 5B & 5C show the specificity of transcriptional activity of the mutant human progesterone receptor.

Figure 6 depicts the transient transfection of mutant human progesterone human receptor into mammalian cells.

15       The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

### DETAILED DESCRIPTION OF THE INVENTION

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

#### Definitions:

25       The term "steroid hormone receptor superfamily" as used herein refers to the superfamily of steroid receptors, some of which are known steroid receptors whose primary sequence suggests that they are related to each other. Representative examples of such receptors include the estrogen, progesterone, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors.

30       Receptors are composed of a DNA binding domain and a ligand binding domain. The DNA binding domain contains the receptor

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regulating sequence and binds DNA and the ligand binding domain binds the specific biological compound (ligand) to activate the receptor.

5 The term "orphan receptors" as used herein refers to a family of approximately twenty receptors whose primary amino acid sequence is closely related to the primary amino acid sequence of the steroid hormone receptor. They are called orphan receptors because no ligand has been identified which directly activates any of the members of this family.

10 "A and B forms of the progesterone receptor" are two distinct forms of the progesterone receptor that are derived from the same gene. The process for generation of the products may be alternate initiation of transcription, splicing differences or may relate to the promotor structure.

15 "Estrogen response element" is a synthetic or naturally occurring DNA sequence which, when placed into a heterologous promotor can confer estrogen responsiveness to that promotor in the presence of estrogen activated estrogen receptor.

The term "ligand" refers to any compound which activates the receptor, usually by interaction with (binding) the ligand binding domain of the receptor. However, ligand can also include compounds which activate the receptor without binding.

20 "Agonist" is a compound which interacts with the steroid hormone receptor to promote a transcriptional response. Example estrogen is an agonist for the estrogen receptor, compounds which mimic estrogen would be defined as steroid hormone receptor agonists.

25 "Antagonist" is a compound which interacts with or binds to a steroid hormone receptor and blocks the activity of a receptor agonist.

The term "non-natural ligands" refer to compounds which are normally not found in animals or humans and which bind to the ligand binding domain of a receptor.

30 The term "anti-hormones" refers to compounds which are receptor antagonists. The anti-hormone is opposite in activity to a hormone.

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The term "non-native ligands" refers to those ligands which are not naturally found in the specific organism (man or animal) in which gene therapy is contemplated. For example, certain insect hormones such as ecdysone are not found in humans. This is an example of a non-native hormone to the human or animal.

Examples of non-natural ligands, anti-hormones and non-native ligands include the following:  $11\beta$ -(4-dimethylaminophenyl)- $17\beta$ -hydroxy- $17\alpha$ -propinyl-4,9-estradiene-3-one (RU38486 or Mifepristone);  $11\beta$ -(4-dimethylaminophenyl)- $17\alpha$ -hydroxy- $17\beta$ -(3-hydroxypropyl)- $13\alpha$ -methyl-4,9-gonadiene-3-one (ZK98299 or Onapristone);  $11\beta$ -(4-acetylphenyl)- $17\beta$ -hydroxy- $17\alpha$ -(1-propinyl)-4,9-estradiene-3-one (ZK112993);  $11\beta$ -(4-dimethylaminophenyl)- $17\beta$ -hydroxy- $17\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one (ZK98734); (7 $\beta$ , $11\beta$ , $17\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2'(3'H)-furan]-3-one (Org31806); (11 $\beta$ , $14\beta$ , $17\alpha$ )-4',5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one (Org31376); 5-alpha-pregnane-3,2-dione.

The term "genetic material" as used herein refers to contiguous fragments of DNA or RNA. The genetic material which is introduced into targeted cells according to the methods described herein can be any DNA or RNA. For example, the nucleic acid can be: (1) normally found in the targeted cells, (2) normally found in targeted cells but not expressed at physiologically appropriate levels in targeted cells, (3) normally found in targeted cells but not expressed at optimal levels in certain pathological conditions, (4) novel fragments of genes normally expressed or not expressed in targeted cells, (5) synthetic modifications of genes expressed or not expressed within targeted cells, (6) any other DNA which may be modified for expression in targeted cells and (7) any combination of the above.

The term "nucleic acid cassette" as used herein refers to the genetic material of interest which can express a protein, or a peptide, or RNA

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after it is incorporated transiently, permanently or episomally into a cell. The nucleic acid cassette is positionally and sequentially oriented in a vector with other necessary elements such that the nucleic acid in the cassette can be transcribed and, when necessary, translated in the cells.

5 "Mutant" refers to an alteration of the primary sequence of a receptor such that it differs from the wild type or naturally occurring sequence. The mutant steroid hormone receptor protein as used in the present invention can be a mutant of any member of the steroid hormone receptor superfamily. For example, a steroid receptor can be mutated by  
10 deletion of amino acids on the carboxy terminal end of the protein. Generally, a deletion of from about 1 to about 120 amino acids from the carboxy terminal end of the protein provides a mutant useful in the present invention. A person having ordinary skill in this art will recognize, however, that a shorter deletion of carboxy terminal amino  
15 acids will be necessary to create useful mutants of certain steroid hormone receptor proteins. For example, a mutant of the progesterone receptor protein will contain a carboxy terminal amino acid deletion of from about 1 to about 60 amino acids. In a preferred embodiment 42 carboxy terminal amino acids are deleted from the progesterone receptor protein.

20 "Null mutation" is a genetic lesion to a gene locus that totally inactivates the gene product.

The term "plasmid" as used herein refers to a construction comprised of extrachromosomal genetic material, usually of a circular duplex of DNA which can replicate independently of chromosomal DNA.  
25 Plasmids are used in gene transfer as vectors. Plasmids which are helpful in the present invention include plasmids selected from the group consisting of UP-1, YEphPR-A879, YEphPR-A891, YEphPR-B891, YEphPR-B879, phPR-A879, phPR-A891, phPR-B879 and phPR-B891.

30 The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic elements positionally and sequentially

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oriented with other necessary elements such that the nucleic acid in a nucleic acid cassette can be transcribed and when necessary translated in the transfected cells. In the present invention the preferred vector comprises the following elements linked sequentially at appropriate distance for allowing functional expression: a promoter; a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal.

As used herein the term "expression vector" refers to a DNA plasmid that contains all of the information necessary to produce a recombinant protein in a heterologous cell.

The term "vehicle" as used herein refers to non-genetic material combined with the vector in a solution or suspension which enhances the uptake, stability and expression of genetic material into targeted cells. Examples of a vehicle include: sucrose, protamine, polybrene, spermidine, polylysine, other polycations, proteins,  $\text{CaPO}_4$  precipitates, soluble and insoluble particles, or matrices for slow release of genetic material. The proteins may be selected from the group including lactoferrin, histone, natural or synthetic DNA binding proteins, natural or synthetic DNA binding compounds, viral proteins, non-viral proteins or any combinations of these. In addition, vehicles may be comprised of synthetic compounds which bind both to DNA and function as ligands for normal receptors on targeted cells.

The term "transformed" as used herein refers to transient, stable or persistent changes in the characteristics (expressed phenotype) of a cell by the mechanism of gene transfer. Genetic material is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products. One skilled in the art readily recognizes that the nucleic acid cassette can be introduced into the cells by a variety of procedures, including transfection and transduction.

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The term "transfection" as used herein refers to the process of introducing a DNA expression vector into a cell. Various methods of transfection are possible including microinjection,  $\text{CaPO}_4$  precipitation, liposome fusion (e.g. lipofection) or use of a gene gun.

5       The term "transduction" as used herein refers to the process of introducing recombinant virus into a cell by infecting the cell with a virus particle. In the present invention, the recombinant virus contains a nucleic acid cassette.

10       The term "transient" as used herein relates to the introduction of genetic material into a cell to express specific proteins, peptides, or RNA, etc. The introduced genetic material is not integrated into the host cell genome or replicated and is accordingly eliminated from the cell over a period of time.

15       The term "stable" as used herein refers to the introduction of genetic material into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. Gene expression after stable transduction can permanently alter the characteristics of the cell leading to stable transformation.

20       The term "persistent" as used herein refers to the introduction of genes into the cell together with genetic elements which enable episomal (extrachromosomal) replication. This can lead to apparently stable transformation of the characteristics of the cell without the integration of the novel genetic material into the chromosome of the host cell.

25       The term "pharmacological dose" as used herein with a vector/molecular switch complex refers to a dose of vector and level of gene expression resulting from the action of the promoter on the nucleic acid cassette when introduced into the appropriate cell type which will produce sufficient protein, polypeptide, or antisense RNA to either  
30       (1) increase the level of protein production, (2) decrease or stop the production of a protein, (3) inhibit the action of a protein, (4) inhibit proliferation or accumulation of specific cell types, or (5) induce

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proliferation or accumulation of specific cell types. The dose will depend on the protein being expressed, the promoter, uptake and action of the protein RNA. Given any set of parameters, one skilled in the art will be able to determine the dose.

5           The term "pharmacological dose" as used herein with a ligand refers to a dose of ligand sufficient to cause either up-regulation or down-regulation of the nucleic acid cassette. Thus, there will be a sufficient level of ligand such that it will bind with the receptor in the appropriate cells in order to regulate the nucleic acid cassette. The specific dose of  
10   any ligand will depend on the characteristics of the ligand entering the cell, binding to the receptor and then binding to the DNA and the amount of protein being expressed and the amount of up-regulation or down-regulation needed. Given any set of parameters, one skilled in the art will be able to determine the appropriate dose for any given receptor being  
15   used as a molecular switch.

"Plasmid activity" is a phenotypic consequence that relates specifically to introduction of a plasmid into an assay system.

"Transcriptional activity" is a relative measure of the degree of RNA polymerase activity at a particular promoter.

20           "Receptor activity" is a phenotypic consequence that relates specifically to introduction of a receptor into an assay system.

The present invention provides mutant steroid hormone receptor proteins. These mutated steroid hormone receptor proteins are capable of distinguishing, and are useful in methods of distinguishing a steroid  
25   hormone receptor antagonist from a steroid hormone receptor agonist.

The present invention further provides plasmids containing mutated steroid hormone receptor proteins. Plasmids of the present invention may contain mutant proteins of any of the hormones in the steroid hormone receptor superfamily.

30           The present invention also provides transfected cells containing plasmids having mutated steroid hormone receptor proteins inserted



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therein. Useful cells for transfection include yeast, mammalian and insect cells.

In a specific embodiment, the yeast is *Saccharomyces cerevisiae*. In a specific embodiment the mammalian cell is selected from the group consisting of HeLa, CV-1, COSM6, HepG2, CHO and Ros 17.2. In a specific embodiment the insect cells are usually selected from the group consisting of SF9, drosophila, butterfly and bee.

The present invention also provides stable cell lines transformed with the plasmids of the present invention.

The plasmids and transfected cells of the present invention are useful in methods of determining whether a compound has antagonist or agonist activity at a steroid hormone receptor. This method comprises contacting a compound of interest with a transfected cell of the present invention. If the compound induces transcription, it has a steroid hormone receptor antagonist. If no transcription is induced, the compound may be a steroid hormone receptor agonist.

The present invention also provides a method of determining an endogenous ligand for a steroid hormone receptor protein. This method comprises initially contacting a compound with a transfected cell of the present invention. Subsequently, the transcription level induced by the compound is measured. The higher the transcription level the more strongly the indication that the compound is an endogenous ligand of the specific receptor being tested.

In addition, the present invention provides endogenous ligands for steroid hormone receptor proteins. An endogenous ligand for a steroid hormone receptor protein is capable of stimulating transcription when in the presence of a transfected cell of the present invention. The endogenous ligand binds to the mutated steroid receptor of the present invention and stimulates transcription in cells containing the mutated receptor.

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Another alternative embodiment of the present invention is a molecular switch for regulating expression of a heterologous nucleic acid sequence in gene therapy.

5 In a preferred embodiment of the present invention, the molecular switch for regulating expression of a heterologous nucleic acid cassette in gene therapy, comprises a modified steroid receptor which includes a natural steroid receptor DNA binding domain attached to a modified ligand binding domain. In the preferred embodiment of the molecular switch the modified binding domain usually binds only ligand compounds  
10 which are non-natural ligands, anti-hormones or non-native ligands. One skilled in the art readily recognizes that the modified ligand binding domain may bind native ligands, but there is insignificant binding and thus very little, if any, regulation.

15 In a preferred embodiment, the modified steroid receptor is a progesterone receptor with the DNA binding domain replaced with a DNA binding domain selected from the group consisting of GAL-4 DNA, virus DNA binding site, insect DNA binding site and a non-mammalian DNA binding site.

20 The molecular switch can be further modified by the addition of a transactivation domain. The transactivation domains which are usually used include VP-16, TAF-1, TAF-2, TAU-1 and TAU-2. One skilled in the art will readily recognize that a variety of other transactivation domains are available.

25 In a preferred embodiment the progesterone receptor has the modified ligand binding domain GAL-4 DNA binding domain and a transactivation domain such as TAF-1.

30 In a further embodiment, the progesterone receptor has the ligand binding domain replaced with an ecdysone binding domain. Again, the function of this molecular switch can be enhanced by adding a TAF-1 transactivation domain.

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One skilled in the art will readily recognize the molecular switch can be made tissue specific by selecting the appropriate transactivation domains, ligand binding domains and DNA binding domains. In particular, one skilled in the art readily recognizes that by adding a transactivation domain which is specific to a given tissue, the molecular switch will only work in that tissue. Also, the addition of a tissue-specific cis-element to the target gene will aid in providing tissue-specific expression.

The present invention also envisions a method of regulating gene expression of a nucleic acid cassette in gene therapy. This method comprises the step of attaching the molecular switch to a nucleic acid cassette used in gene therapy. In the preferred embodiment, the nucleic acid sequence which is expressed is heterologous. The combined nucleic acid cassette/molecular switch is then administered in a pharmacological dose to an animal or human to be treated or to a transgenic animal or to a plant.

One skilled in the art readily appreciates that the combined nucleic acid cassette/molecular switch can be introduced into the cell in a variety of ways both *in vivo* and *ex vivo*. The introduction can be by transfection or transduction. After the nucleic acid cassette/molecular switch is introduced into the cell, the cassette in the resultant transformed cell can be either up-regulated (turned on) or down-regulated (turned off) by introducing to the animal or human a pharmacological dose of a ligand which binds the modified ligand binding site.

In one embodiment of the present invention there is a method for regulating nucleic acid cassette expression in gene therapy comprising the step of linking a molecular switch to a nucleic acid cassette. This molecular switch/nucleic acid cassette is introduced into a cell to form a transformed cell. The transformed cell is then inserted in a pharmacological dose into a human or animal for gene therapy.

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In another embodiment the molecular switch/nucleic acid cassette is directly injected into a targeted cell *in vivo* for gene therapy.

For example, in the treatment of senile dementia or Parkinson's disease, the nucleic acid cassette within the nucleic acid cassette contains a growth factor, hormone or neurotransmitter and the cell is a brain cell. In a preferred embodiment the naked brain cell containing the cassette can be encapsulated in a permeable structure. The naked brain cell or the permeable structure containing the brain cell is then inserted into the animal or human to be treated. The permeable structure is capable of allowing the in/out passage of activators of the molecular switch and growth factors but prevents the passage of attack cells that would interact with and damage the implanted brain cells. In the preferred embodiment it is important to encapsulate the brain cells, since introduction of naked brain cells often results in attack by the body's defense system and the destruction of these cells. One skilled in the art recognizes that a variety of encapsulation procedures and structures are available in the art.

In the treatment of senile dementia or Parkinson's disease, it is found that the molecular switch in the preferred embodiment includes a progesterone receptor with the modified ligand binding domain replaced attached to a GAL-4 DNA. A growth factor is produced in the transformed cell by giving a pharmacological dose of an appropriate ligand to turn the molecular switch on (up-regulation) to the animal or human to be treated. For example, an anti-progesterone such as RU38486 can be given. The amount of growth factor produced is proportional to the dose of ligand given. One skilled in the art will be able to determine a pharmacological dose depending on the molecular switch used and the ligand used.

Another embodiment of the present invention employs a dual system of agonist/antagonist pairs. In this system a custom up-regulation ligand is chosen and the desired receptor mutation or modified receptors are made. Then a second round of ligand screening and mutation is

performed to develop a receptor which also binds a specific, selective down-regulator ligand. In the preferred embodiment the ligands share a normal metabolic clearance pathway of the host's endogenous ligands, thereby avoiding problems of toxicity and long half-life. In the screening process either yeast, animal or insect cells can be used. In the preferred embodiment yeast cells are used.

In addition to selecting transactivation elements and receptors for tissue specificity, one skilled in the art also recognizes that tissue specificity can be achieved with specific ligands. For example, ligands can be chosen which act only in certain tissues due to requirements for terminal conversion to active metabolites. A synthetic androgen which binds a transfected androgen receptor is made. This androgen, however, requires metabolism to the 5-alpha reduced form to be active. In this manner only classical androgen end-organs are able to metabolize the new ligand to its proper chemical form. Other cells of the body lacking the 5-alpha reductase will not activate the transgene via this compound.

Alternatively, a ligand which is active only when it is not further metabolized to the 5-alpha reduced form is used. In this case, the ligand would be active only in classical androgen end-organ cells. Since 5-alpha reductase inhibitors are currently available therapeutic agents, they can be used in conjunction with the present invention to allow complete shutdown or complete activation of the receptor bypassing the ligand route if some sort of emergency required that approach.

Side chains are usually tolerated at certain positions on ligands of the receptor superfamily. For example, the 7-alpha position of certain ligands, such as estradiol and progesterone, can be attached to sidechains and the ligands will still bind to receptors. Suitable sidechains can be used to either increase or restrict solubility, membrane transfer or target organ accessibility. Thus, even specific ligands can be made to show tissue preference. For example, the synthetic steroid R5020 (17 $\alpha$ , 21-dimethyl-19-Nor-pregn-4,9-diene-3,20-dione) does not enter tissue culture cells at

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low temperatures at which progesterone enters freely. One skilled in the art readily recognizes that other modifications can be made to ligands to tailor their use as up- or down-regulating agents in the present invention.

5 The following samples are offered by way of illustration and are not intended to limit the invention in any way.

#### EXAMPLE 1

The homogenization buffer for hormone binding assays contained 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4 (TESH buffer). The homogenization buffer for Western analysis of receptor  
10 contained 10 mM Tris-HCl, 2 mM EDTA, 45 mM dithiothreitol, 10% glycerol and 300 mM NaCl (TEDG + salts).

#### Yeast strain

The *Saccharomyces cerevisiae* strain BJ3505 (MAT $\alpha$ , pep4:HIS3, prb1- $\Delta$ 1.6R, his3 $\Delta$ 200, lys2-801, trp1- $\Delta$ 101, ura3-52, gal2, (CUP1)) was used  
15 (Yeast Genetic Stock Center, Berkeley, CA). All yeast transformations were carried out following the lithium acetate transformation protocol (Ito, et al., *J. Bacteriol.* 153:163-168, 1983).

The PCR reactions were carried out using YEphPR-B DNA template (a YEp52AGSA-derived yeast expression plasmid containing the  
20 cDNA of hPR form-B (Misrahi, et al., *Biochem. Bioph. Res. Comm.* 143:740-748, 1987) inserted downstream of the yeast methallothionein-CUP1 promoter) and using three different sets of primers. In order to decrease the fidelity of the second strand polymerization reaction, buffer conditions of 1.5 mM MgCl<sub>2</sub>, 0.1 mM dNTPs and pH 8.2 were used. About  
25 2000 primary transformants were obtained from each region-specific library.

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**EXAMPLE 2****Yeast Mutant Screening**

Colonies of each library of hPR molecules mutated in specific subregions were pooled, large amounts of DNA were prepared and used to transform yeast cells carrying the reporter plasmid YRpPC3GS+, which contains two GRE/PRE elements upstream of the CYC1 promoter linked to the Lac-Z gene of *E. coli* (Mak, et al., *J. Biol. Chem.* 265:20085-20086, 1989). The transformed cells were plated on 1.5% agar plates containing 2% glucose, 0.5% casamino acids (5% stock solution of casamino acids is always autoclaved before use to destroy tryptophan), 6.7 g/l yeast nitrogen base (without amino acids) and 100  $\mu$ M CuSO<sub>4</sub> (CAA/Cu plates) and grown for 2 days at 30°C. These colonies were then replica-plated on CAA/Cu plates containing 0.16 g/l of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal, an indicator of  $\beta$ -galactosidase activity) with or without the hormones as indicated in Fig. 1 and allowed to grow for one day at 30°C, then two days at room temperature in the dark.

**EXAMPLE 3****Growth of Yeast Culture for *in vitro* Assay**

*Saccharomyces cerevisiae* cells containing YEphPRB and the reporter plasmid were grown overnight at 30°C in minimal media containing 2% glucose. The cells were subcultured in fresh medium and allowed to grow until early mid-log phase (O.D.<sub>600nm</sub>=1.0). Induction of receptor was initiated by the addition of 100  $\mu$ M copper sulfate to the culture. Cells were harvested by centrifugation at 1,500 xg for 10 minutes and resuspended in the appropriate buffer. This and all subsequent steps of analysis of the yeast extracts were done at 4°C.

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**EXAMPLE 4****Transcription Assay**

Yeast cells containing the reporter and expression plasmids were grown overnight as described above in Example 3 in the presence of 100  $\mu$ M copper sulfate. When the cell density reached O.D.<sub>600nm</sub> = 1.0, hormones were added to the cultures. After a 4 hour incubation, yeast extracts were prepared and assayed for  $\beta$ -galactosidase activity as described previously (Miller, *J. M. Miller ed.*, 352-355, 1972).

Generally, reporters useful in the present invention are any which allow for appropriate measurement of transcription levels. Preferable reporter systems include reporter vectors comprised of the yeast iso-1-cytochrome C proximal promoter element fused to a structural gene, wherein said structural gene is selected from the group consisting of  $\beta$ -galactosidase, galactokinase and URA3. More preferably, the vector is comprised of an insertion site for a receptor response element. The vectors which include  $\beta$ -galactokinase as an indicator of transcriptional activity are derived from the parent vector PC2 while the vectors which include galactokinase are derived from YCpR1 vector. Preferably, the structural genes originate from *E. coli*.

**EXAMPLE 5****Western Immunoblotting**

Yeast cells were grown as described in Example 4 for the transcription assay. Yeast extracts for Western blot analysis were prepared by resuspending the cell pellet in TEDG+salts. The cell suspension was mixed with an equal volume of glass beads and disrupted by vortexing in a microcentrifuge tube. The homogenate was centrifuged at 12,000 x g for 10 minutes. The supernatant was collected and the protein concentration was estimated using bovine serum albumin as standard. Yeast extracts were resolved on a 0.1% sodium dodecyl sulfate-7% polyacrylamide gel and transferred to Immobilon membrane as



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described previously (McDonnell, et al., *Mol. Cell. Biol.* 9:3517-3523, 1989). Solid phase radioimmunoassay was performed using a monoclonal antibody (JZB39) directed against the N-terminal domain of A and B forms of hPR.

5

**EXAMPLE 6****Hormone Binding Competition Assays**

Induction of PR synthesis was initiated by the addition of 100  $\mu$ M CuSO<sub>4</sub> to the culture and incubation was continued for 6 hours. The cell pellet was resuspended in TESH buffer containing 1  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml PMSF and 10  $\mu$ g/ml pepstatin. The cell suspension was mixed with an equal volume of glass beads (0.5 mm; B. Braun Instruments) and disrupted by vortexing in a microcentrifuge tube. The homogenate was centrifuged at 12,000 x g for 10 minutes and the supernatant was further centrifuged at 100,000 x g for 30 minutes to obtain a cytosol fraction. Diluted yeast extracts (200  $\mu$ l) containing 100  $\mu$ g of total protein were incubated overnight at 4°C with [<sup>3</sup>H]ligand in the absence (total binding) or presence (non-specific binding) of a 100-fold excess of unlabelled ligand. Bound and free steroids were separated by addition of 500  $\mu$ l of dextran-coated charcoal suspension (0.5% Norit A, 0.05% dextran, 10 mM Tris HCl, pH 7.4 and 1 mM EDTA). Specific binding was determined by subtracting nonspecific from total binding. Scatchard analysis was carried out as described previously by Mak, et al., *J. Biol. Chem.* 264:21613:21618 (1989).

25

**EXAMPLE 7****Site-directed mutagenesis**

Mutants YEphPR-B879 and YEphPR-B891 were prepared following the procedure described by Dobson, et al., *J. Biol. Chem.* 264:4207-4211 (1989). CJ236 cells were infected with mpPR90 (an M13 plasmid containing hPR cDNA). The resulting uridine-containing single-stranded

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DNA was annealed to 20-mer oligonucleotides containing a TGA stop codon corresponding to amino acids 880 and 892, respectively.

#### EXAMPLE 8

##### Construction of Mammalian Expression Vectors

5           The mammalian expression vector phPR-B contains the SV40 enhancer sequence upstream of the human growth hormone promoter linked to the hPR-B cDNA. This vector was digested with SalI and EcoRI. The 6.1kb fragment (containing the vector sequences and the 5'-1.5 kb of the hPR) was gel-purified and ligated to the 2.1 kb fragment of  
10   YEphPR-B891 (containing the 3'-end of the receptor). previously cut with SalI and EcoRI. The resulting plasmid, phPR-B891, encodes a 42 amino acid truncated version of hPR form B.

#### EXAMPLE 9

##### Mammalian Cell Transient Transfections and CAT-Assays

15           Five  $\mu$ g of chloramphenicol acetyltransferase (CAT) reporter plasmid, containing two copies of a PRE/GRE from the tyrosine amino transferase gene linked to the thymidine kinase promoter (PRETKCAT), were used in transient cotransfection experiments together with 5  $\mu$ g of wild type or mutant receptor DNAs. Transient cotransfections and CAT-  
20   assays were performed as described by Tsai, et al., *Cell* 57:443-448 (1989).

#### EXAMPLE 10

##### Mutagenesis of the hormone binding domain of hPR-B

25           In order to characterize amino acids within the hPR HBD which are critical for ligand binding and hormone-dependent transactivation, libraries of mutated hPR molecules were created and the mutants introduced into a reconstituted progesterone-responsive transcription system in yeast. This system allowed the screening of large numbers of mutant clones and the direct, visual identification of phenotypes.

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Unique restriction sites for *NaeI*, *AvrII* and *EcoNI* were created in the cDNA of hPR, obtaining three cassettes of 396, 209 and 400 nucleotides (regions 1, 2 and 3, respectively). For PCR mutagenesis three sets of primers (16 + 7 for region 1, 5 + 4 for region 2 and 6 + 13 for region 3) were used in the polymerization reaction using YEphPR-B as DNA template. The fragments obtained after PCR were digested with the appropriate enzymes, gel-purified and ligated into the parental plasmid YEphPR-B. Ligation mixes were used to transform bacterial cells and to obtain libraries of hPR molecules randomly point-mutated in the HBD. 5  $\mu$ g of DNA were used from each library to transform yeast cells carrying the reporter plasmid YRpPC3GS+ and transformants were selected for tryptophan and uracil auxotrophy on CAA plates containing 100  $\mu$ M CuSO<sub>4</sub>. These were then replicated on CAA plates containing the hormones. The screening for "up-mutations" allowed identification of receptor mutants with hormone-independent transcriptional activity, or increased affinity for the ligand (these clones should remain blue when grown with 100-fold less hormone), or with an altered response to RU38486 or a glucocorticoid analogue. In the "down-mutation" screening, receptor mutants that were transcriptionally inactive in the presence of the ligand were detected.

Because of the nature of the method used to generate the mutated DNA templates, it was necessary, firstly, to determine the quality of the libraries obtained. This was assessed by estimating the number of null-mutations generated by mutagenesis. We estimated the frequency of occurrence of transcriptionally inactive receptors (white colonies) compared to the total number of colonies. This frequency was about 7%.

The primary transformants were replica-plated onto plates containing the antiprogesterin RU38486. The wild type receptor is not activated by this hormone (Fig. 1). Using this screening strategy, a single colony was identified that displayed considerable transcriptional activity in response to the antihormone. Interestingly, the same colony did not

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display transcriptional activity when replica-plated in the presence of progesterone. The colony was purified and the phenotype was confirmed. Eviction of the expression vector from the clone, followed by reintroduction of the unmutated receptor, demonstrated that the phenotype was indeed related to the expression vector and was not the result of a secondary mutation. In addition, the mutated plasmid called UP-1, was rescued from yeast by passage through *E.coli* (as described in Ward, *Nucl. Acids Res.* 18:5319 (1990) and purified. This DNA was then reintroduced into yeast that contained only the reporter plasmid. As expected, the mutant phenotype was stable and related directly to the receptor expression plasmid.

#### EXAMPLE 11

##### Characterization of the UP-1 mutant

The plate assays used to identify the receptor mutants are qualitative in nature. To further characterize the properties of UP-1, the activity of the receptor mutants was compared with that of the wild type receptor in a transcription assay. In this method, yeast cells transformed with either the wild type or the mutant receptor and a progesterone responsive reporter were grown overnight in the presence of 100  $\mu$ M CuSO<sub>4</sub>. When the cells had reached an O.D.<sub>600nm</sub> of 1.0, they were supplemented with progesterone or RU38486 and harvested by centrifugation after four hours. The  $\beta$ -galactosidase activity in the cell cytosol was then measured.

With reference to Figure 2A, when assayed with the wild type receptor, 1  $\mu$ M RU38486 is a weak inducer of transcription, whereas progesterone caused a greater than 60-fold induction of transcription at 1  $\mu$ M. In Figure 2B however, this situation was reversed when the mutant was analyzed. In this case, RU38486 was an extremely potent activator, whereas progesterone was ineffective. Interestingly, the activity achieved by the mutant in the presence of RU38486 was of the same order

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of magnitude as that of the wild type assayed in the presence of progesterone. This reversal in specificity clearly indicates that the mechanism by which these ligands interact with the receptor is basically different.

5           Figure 2C shows the DNA and amino acid sequences of the wild type and mutant DNAs. The cytosine at position 2636 was missing in the mutant DNA, therefore, a shifted reading frame was created and a stop codon was generated 36 nucleotides downstream of the C-2636 deletion. A schematic structure of the wild type and UP-1 receptors is also  
10           presented with a depiction of the 12 C-terminal amino acids unique to the mutant receptor. Conserved and structurally similar amino acids are marked by an apostrophe and asterisk, respectively.

          DNA sequence analysis of UP-1 identified a single nucleotide deletion at base 2636 (Fig. 2C). This mutation results in a shift of the  
15           reading frame which generates a stop codon 36 nucleotides downstream. As a result, the wild type receptor is truncated by 54 authentic amino acids and 12 novel amino acids are added at the C-terminus.

### **EXAMPLE 12**

#### **Western Analysis of the Mutant Human Progesterone Receptor**

20           Figure 3 shows a western analysis of mutant hPR. Yeast cells carrying the reporter plasmid and wild type (yhPR-B\_ or mutant (UP-1) hPR were grown overnight in CAA medium with (lanes 3 to 5 and 7 to 9) or without (lanes 2 and 6) 100  $\mu$ M CuSO<sub>4</sub>. 1  $\mu$ M progesterone or 1  $\mu$ M RU38486 were added as indicated and cells were grown for another 4  
25           hours. Yeast extracts were prepared as described above. 50  $\mu$ g of protein extract were run on a 0.1% SDS-7% polyacrylamide gel. 50  $\mu$ g of a T47D nuclear extract containing the A and B forms of hPR were also loaded (lane 1) as a positive control. The positions of molecular weight markers are indicated.

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A Western immunoblot analysis of UP-1 and wild type receptors was performed in order to verify that the mutant receptor was synthesized as predicted from its DNA sequence and to eliminate the possibility that some major degradation products were responsible for the mutant phenotype. As shown in Fig. 3, the mutant receptor migrated faster in the gel, confirming the molecular weight predicted by DNA sequencing. The wild type receptor (yhPR-B) ran as a 114 kDa protein, while the mutant receptor was 5kDa smaller (compare lanes 2 and 3 with 6 and 7). The addition of 100 $\mu$ M CuSO<sub>4</sub> to the cell cultures increased synthesis of both the wild type and mutant hPR to the same extent. No major degradation products were detected. In the presence of progesterone and RU38486, yhPR-B bands were upshifted due to hormone-induced phosphorylation of the receptor. In contrast, RU38486 induced upshifting of wild type PR to a lesser extent (lanes 4 and 5). For the UP-1 mutant this hormone-dependent upshifting was seen upon treatment with RU38486 (lanes 8 and 9). Thus, the C-terminus of PR may be responsible for the inactivity of RU38486. Consequently, removal of this sequence would enable RU38486 to become an agonist.

### EXAMPLE 13

#### Hormone binding analysis

Figure 4 shows the transcriptional activity and hormone binding analysis of wild type and mutant hPR constructs. hPR constructs are reported to the left side together with a schematic representation of the receptor molecules. Yeast cells were grown in the presence of 100  $\mu$ M CuSO<sub>4</sub>. Transcriptional analysis was done as described above. Experiments were done in triplicate and transcriptional activities were normalized with respect to protein. Hormone binding assays were performed in the presence of 20 nM [<sup>3</sup>H] progesterone or 20 nM [<sup>3</sup>H] RU38486.

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A saturation binding analysis of the UP-1 mutant receptor was performed in order to determine if its affinity for RU38486 and progesterone was altered. Scatchard analysis of the binding data demonstrated that both the wild type and mutant receptors had a similar affinity for RU38486 of 4 and 3 nM, respectively. As seen in Figure 4, the mutant receptor molecule had lost the ability to bind progesterone. Thus, the amino acid contacts for progesterone and RU38486 with hPR are different.

#### Generation of deletion mutants of hPR-B

As shown in Fig. 2C, DNA sequencing revealed that the frameshift mutation in the UP-1 clone created a double mutation in the receptor protein. That is, a modified C-terminal amino acid sequence and a 42 amino acid truncation. In order to identify which mutation was ultimately responsible for the observed phenotype, two new receptor mutants were constructed *in vitro*: YEphPR-B879, containing a stop codon corresponding to amino acid 880, and YEphPR-B891, containing a stop codon at amino acid 892. Hormone binding data (see Fig.4) demonstrated that both of these truncated receptors could bind RU38486 but not progesterone. When examined *in vivo*, both mutant receptors activated transcription in the presence of RU38486 to levels comparable to those of the mutant UP-1 generated in yeast. As expected, both mutants were inactive in the presence of progesterone. Thus, the observed phenotype was not due to second site mutations in the UP-1 molecule. Also, 12 additional amino acids, from 880 to 891, were not responsible for the mutant activity. In addition, it is clear the C-terminal 42 amino acids are required for progesterone to bind to the receptor while the last 54 amino acids are unnecessary for RU38486 binding. Thus, the antagonist is contacting different amino acids in the native receptor molecule and may induce a distinct receptor conformation relative to agonists.

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**EXAMPLE 14****Steroid specificity for activation of transcription of the UP-1 mutant**

Figure 5 shows the specificity of the transcriptional activity of the mutant hPR. In panel (A), wild type and UP-1 mutant receptor transcriptional activities were assayed in the presence of different concentrations of progesterone, RU38486, Org31806 and Org31376 as indicated.

A transcription assay was performed using two synthetic antagonists, Org31806 and Org31376, which are potent antiprogestins. As shown in Fig. 5A, the mutant receptor was activated by both of these compounds. The curve of the concentration-dependent activity was similar to that obtained with RU38486, suggesting that the affinity of these two antagonists for the mutant receptor is similar to that of RU38486. When assayed with the wild type receptor, these compounds had minimal transcriptional activity and behaved like partial agonists (3-10% of progesterone activity) only at concentrations of 1  $\mu$ M, as does RU38486. Thus, the inhibitory effect of the C-terminus of hPR extends to other receptor antagonists.

In panel (B), transcriptional activities of wild type and UP-1 mutant receptors were assayed in the presence of 1  $\mu$ M progesterone (P), RU38486 (RU), R5020 (R), dexamethasone (D), cortisol (C), estradiol (E), tamoxifen (TX) or nafoxidine (N) (see Fig.5B). The synthetic agonist R5020 had no effect on the UP-1 mutant, suggesting that agonists, such as progesterone and R5020, require the C-terminus of the native receptor for binding and consequently fail to recognize the truncated UP-1 receptor. Other steroids known to enter yeast cells, such as estradiol, the antiestrogens tamoxifen and nafoxidine, dexamethasone and cortisol, might possibly activate the mutated receptor. All steroids tested were found to be inactive with either the wild type or mutant receptor. Thus, the activation of the mutant receptor is specific to antiprogestins.



**EXAMPLE 15****Transcriptional activity of mutant receptors in mammalian cells**

Figure 6 shows the transient transfection of mutant hPR into mammalian cells. In panel (A), HeLa cells were transiently transfected with phPR-B and pHPR-B891 receptors together with PRETKCAT receptor plasmid using the polybrene method of transfection as described (Tsai, et al. 1989). Cells were grown with or without 100 nM progesterone or RU38486 for 48 hours prior to harvesting. CAT assays were performed as described above. In panel (B), CV-1 cells were transiently transfected as in (A).

With reference to Figure 6, mutant receptor activity was assayed in both human endometrial HeLa cells and monkey kidney CV-1 fibroblasts. A mutant, phPR-891, was constructed by replacing the full-length PR insert of phPR-B vector with the truncated PR cDNA of YEphPR-B891. The resulting receptor mutant, phPR-B891, is a 42 amino acid truncation of hPR-B form. Mutant 891 and wild type receptors were transfected into HeLa cells together with the PRETKCAT reporter plasmid, which contains two copies of a GRE/PRE element.

As expected, wild type PR activated transcription of the CAT gene reporter in the presence of  $10^{-7}$ M progesterone (Fig. 6A). Although basal transcription level was high, a 3- to 4-fold induction of transcription was detected when progesterone was added to the media. In contrast, no induction occurred in the presence of RU38486. The high basal level of transcription detected in these experiments may mask or alter an RU38486 effect on wild type hPR.

On the other hand, an induction of CAT activity was observed when the 891 mutant was incubated in the presence of  $10^{-7}$ M RU38486 (Fig. 6A). The same concentration of progesterone had no activity.

Cell-type specific factors can influence the activity of the transactivating domains of steroid receptors. In order to evaluate this possibility, wild type and mutant receptors were transfected into CV-1

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cells. Similar results were obtained, i.e., progesterone activated the wild type receptor while RU38486 activated 891 mutant receptor (Fig. 6B).

5 The protein synthesized from phPR-B891 plasmid was of the correct molecular weight in mammalian cells. The mutant receptor was transfected into COSM6 cells. Western analysis on cell extracts showed that the 891 mutant was synthesized, as expected, as a protein of 109 kDa, which corresponds to a protein 42 amino acids shorter than the wild type hPR. Thus, RU38486 acts as an agonist of the truncated B-receptor in a yeast reconstituted system and also in mammalian cells. The mechanism  
10 of transactivation does not require the C-terminal tail of the mutant receptor and is conserved between the three species tested.

#### EXAMPLE 16

##### Chicken progesterone

15 Chicken and mammalian progesterone are readily available and both function by binding to the same DNA regulatory sequence. Chicken progesterone, however, binds a different spectrum of ligands, possessing different affinities from those interacting with human progesterone. Thus, the chicken progesterone can be used as a transgene regulator in humans. Further, it can be used to screen for specific ligands which activate  
20 chicken progesterone but not endogenous human progesterone. An example of a ligand is 5-alpha-pregnane-3,20-dione (dihydroprogesterone) which binds extremely well to chicken progesterone but does not bind or binds very poorly to human progesterone.

25 Although the unmodified chicken progesterone is already endowed with a different spectrum of ligand affinity from the human or other mammals and can be used in its native form, it is important to try to select additional mutated progesterones to create a more efficacious receptor. The differences in chicken and human progesterone receptors are due to a few amino acid differences. Thus, other mutations could be  
30 artificially introduced. These mutations would enhance the receptor

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differences. Screening receptor mutations for ligand efficacy produces a variety of receptors in which alterations of affinity occur. The initial screening of progesterone mutants was carried out using intermediate levels of ligands. One mutant had lost progesterone affinity entirely, but  
5 bound a synthetic ligand RU38486 with nearly wild-type efficiency. RU38486 is normally considered an antagonist of progesterone function, but had become an agonist when tested using this specific mutant. Because the ligand is synthetic, it does not represent a compound likely to be found in the humans or animals to be treated with gene therapy.  
10 Although RU38486 works as an agonist in this case, it is not ideal because of its potential side effects as an anti-glucocorticoid. Further, it also binds to the wild-type human progesterone. Thus it has the undesirable side effect of reproductive and endocrine disfunction.

This approach is not limited to the progesterone receptor, since it  
15 is believed that all of the ligand activated transcription factors act through similar mechanisms. One skilled in the art recognizes that similar screening of other members of the steroid superfamily will provide a variety of molecular switches. For example, the compound 1,25-dihydroxy-Vitamin D<sub>3</sub> activates the Vitamin D receptor but the compound 24,25-dihydroxy-Vitamin D does not. Mutants of the Vitamin D receptor can be  
20 produced which are up-regulated when bound to 24,25-dihydroxy-Vitamin D, but which no longer up-regulates when bound to 1,25-D<sub>3</sub>.

One skilled in the art recognizes that the ligands are designed to be physiologically tolerated, easily cleared, non-toxic and have specific effects  
25 upon the transgene system rather than the entire organism.

#### **EXAMPLE 17**

##### **Transgenic Animals**

A molecular switch can be used in the production of transgenic animals. A variety of procedures are known for making transgenic  
30 animals, including that described in Leder and Stewart, U.S. Patent No. 4,736,866 issued April 12, 1988 and Palmiter and Bannister Annual

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Review of Genetics, v. 20, pp. 465-499. For example, the UP-1 molecular switch can be combined with the nucleic acid cassette containing recombinant gene to be expressed. For example, lactoferrin can be placed under the control of a basal thymidine kinase promoter into which has been placed progesterone responsive elements. This vector is introduced into the animal germ lines, along with the vector constitutively expressing the UP-1 receptor. The two vectors can also be combined into one vector. The expression of the recombinant gene in the transgenic animal is turned on or off by administering a pharmacological dose of RU 38486 to the transgenic animal. This hormone serves to specifically activate transcription of the transgene. The dose can be adjusted to regulate the level of expression. One skilled in the art will readily recognize that this protocol can be used for a variety of genes and, thus, it is useful in the regulation of temporal expression of any given gene product in transgenic animals.

#### **EXAMPLE 18**

##### **Plants**

In this instance, an UP-1 molecular switch can be attached to the nucleic acid cassette containing the gene or recombinant gene to be expressed. For example, the gene for  $\Delta 9$  desaturase can be placed under the control of a basal 3-phosphoglycerate promoter into which has been placed one or more copies of the progesterone response elements. This vector is then introduced into the plant germ line, along with a vector constitutively expressing the UP-1 receptor. Again, these two vectors can be combined into one vector. At the required time for expression of the nucleic acid cassette, a pharmacological dose of RU 38486 is introduced to activate the transcription. The dose can be adjusted to regulate the level of expression. Thus, one skilled in the art will readily recognize that a variety of genes can be turned on and off in plants by the use of the invention of the present application.

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All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are incorporated herein by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Transformed cells, vectors, compositions, molecular switches and receptors, along with the methods, procedures, treatments and molecules described herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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- (iii) NUMBER OF SEQUENCES: 4
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- 20 (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 25 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: US 07/939,246  
(B) FILING DATE: 02-SEP-1992  
(C) CLASSIFICATION:
- 30 (viii) ATTORNEY/AGENT INFORMATION:  
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-37-

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## 5 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (x) PUBLICATION INFORMATION:

- 15 (H) DOCUMENT NUMBER: US 07/882,771
- (I) FILING DATE: 14-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACTTGCATG ATCTTGTCAA ACAACTTCAT CTGTACTGCT TG

42

## (2) INFORMATION FOR SEQ ID NO:2:

## 20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

-38-

## (x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: US 07/882,771

(I) FILING DATE: 14-MAY-1992

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

5 AATTGCATGA TCTTGTCAAA CAACTTCATC TGTACTGCTT GA 42

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## 15 (x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: US 07/882,771

(I) FILING DATE: 14-MAY-1992

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 Asn Leu His Asp Leu Val Lys Gln Leu His Leu Tyr Cys Leu  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide



-39-

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: US 07/882,771

(I) FILING DATE: 14-MAY-1992

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Cys Met Ile Leu Ser Asn Asn Phe Ile Cys Thr Ala

1

5

10

## WHAT IS CLAIMED IS:

CLAIMS

1. A mutated steroid hormone receptor protein, wherein said receptor protein is capable of distinguishing a hormone antagonist from an agonist.

2. The mutated steroid hormone receptor protein of Claim 1, wherein said receptor protein is selected from the group consisting of estrogen, progesterone, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , mineralocorticoid, androgen thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors.

3. The mutated steroid receptor protein of Claim 1, wherein said protein is mutated by deletion of carboxy terminal amino acids.

4. The mutated steroid hormone receptor protein of Claim 3, wherein said deletion comprises from about 1 to about 120 amino acids.

5. The mutated steroid hormone receptor protein of Claim 3, wherein said protein is mutated by deletion of from about one to about 60 amino acids on the carboxy terminal end of said protein.

6. The mutated steroid hormone receptor protein of Claim 3, wherein said carboxy terminal deletion comprises deletion of 42 amino acids.

7. A plasmid containing the mutated steroid hormone receptor protein of Claim 1.

8. The plasmid of Claim 7, wherein said steroid hormone receptor protein is selected from the group consisting of estrogen, progesterone, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors.

9. The plasmid of Claim 7, designated UP-1.

10. The plasmid of Claim 7, wherein said plasmid is selected from the group consisting of YEphPR-A879, YEphPR-A891, YEphPR-B891, YEphPR-B879, phPR-A879, phPR-A891, phPR-B879 and phPR-B891.

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11. A transfected cell containing the plasmid of Claim 7.
12. The transfected cell of Claim 11, wherein said cell is selected from the group consisting of yeast, mammalian and insect cells.
13. The transfected cell of Claim 12, wherein said yeast is *Saccharomyces cerevisiae*.
14. The transfected cell of Claim 12, wherein said mammalian cell is selected from the group consisting of HeLa, CV-1, COSM6, HepG2, CHO and Ros 17.2.
15. The transfected cell of Claim 12, wherein said insect cell is selected from the group consisting of SF9, drosophila, butterfly and bee.
16. A transformed cell line containing the plasmid of Claim 7.
17. A transfected cell containing the plasmid of Claim 9.
18. A transformed cell line containing the plasmid of Claim 9.
19. A method of determining antagonist activity of a compound for a mutated steroid hormone receptor, comprising the steps of:
  - contacting said compound with a transfected cell of Claim 10; and
  - measuring transcription levels induced by said compound.
20. A method of determining agonist activity of a compound for a mutated steroid hormone receptor comprising the steps of:
  - contacting said compound with transfected cells of Claim 10; and
  - measuring transcription levels induced by said compound.
21. The method of Claim 19 or 20, wherein said receptor protein is selected from the group consisting of estrogen, progesterone, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors.
22. A method of determining an endogenous ligand for a mutated steroid hormone receptor, comprising the steps of:

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contacting a compound with the transfected cells of Claim 11; and

measuring transcription levels induced by said compound.

23. A method of determining an endogenous ligand for a mutated steroid hormone receptor, comprising the steps of:

contacting a compound with the transformed cells of Claim 16; and

measuring transcription levels induced by said compound.

24. A method of determining an endogenous ligand for a mutated steroid hormone receptor, comprising the steps of

contacting a compound with the transfected cells of Claim 17; and

measuring transcription levels induced by said compound.

25. A method of determining an endogenous ligand for a mutated steroid hormone receptor, comprising the steps of:

contacting a compound with the transformed cells of Claim 18; and

measuring transcription levels induced by said compound.

26. An endogenous ligand for a mutated steroid hormone receptor, wherein said ligand is capable of binding the mutated steroid hormone receptor and stimulating transcription in the transfected cells of Claim 11.

27. An endogenous ligand for a mutated steroid hormone receptor, wherein said ligand is capable of binding the mutated steroid hormone receptor and stimulating transcription in the transformed cells of Claim 16.

28. An endogenous ligand for a mutated steroid hormone receptor, wherein said ligand is capable of binding the mutated steroid

-43-

hormone receptor and stimulating transcription in the transfected cells of Claim 17.

29. An endogenous ligand for a mutated steroid hormone receptor, wherein said ligand is capable of binding the mutated steroid hormone receptor and stimulating transcription in the transformed cells of Claim 18.

30. A mutated progesterone receptor protein, said mutated progesterone receptor having a carboxy terminal deletion of 42 amino acids.

31. A composition of matter comprising plasmid UP-1 containing a mutated steroid hormone receptor.

32. A molecular switch for regulating expression of a nucleic acid cassette in gene therapy, comprising:

a modified steroid receptor, said receptor including a natural steroid receptor DNA binding domain linked to a modified ligand binding domain,

33. The molecular switch of claim 32, wherein the natural steroid receptor DNA binding domain has been replaced with a non-native or modified DNA binding domain.

34. The molecular switch of claim 32 or 33, wherein the ligand binding domain is modified to bind a compound selected from the group consisting of non-natural ligands, anti-hormones and non-native ligands.

35. The molecular switch of claim 32 or 33, wherein the ligand binding domain binds a compound selected from the group consisting of 5-alpha-pregnane-3,2-dione; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -propinyl-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadiene-3-one; 11 $\beta$ -(4-acetylphenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(1-propinyl)-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one; (7 $\beta$ ,11 $\beta$ ,17 $\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2'(3'H)-furan]-3-one; (11 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-4',5'-

dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one.

36. The molecular switch of claim 32 or 33, wherein the receptor is selected from the group consisting of estrogen, progesterone, androgen, Vitamin D, COUP-TF, cis-retonic acid, Nurr-1, thyroid hormone, mineralocorticoid, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$  and orphan receptors.

37. The molecular switch of claim 33, wherein the modified steroid receptor is a receptor with the natural DNA binding domain replaced with a DNA binding domain selected from the group consisting of GAL-4 DNA, virus DNA binding site, insect DNA binding site and a non-mammalian DNA binding site.

38. The molecular switch of claim 32 or 33, further comprising linking a transactivation domain selected from the group consisting of VP-16, TAF-1, TAF-2, TAU-1 and TAU-2 to the modified steroid receptor.

39. The molecular switch of claim 32 or 33, wherein the modified receptor is a progesterone receptor with the ligand binding domain replaced with modified ligand binding domain which binds non-natural or non-native ligands.

40. The molecular switch of claim 32 or 33, further comprising a TAF-1 transactivation domain linked to the modified receptor.

41. The molecular switch of claim 32 or 33, wherein the steroid hormone includes an ecdysone ligand binding domain.

42. The molecular switch of claim 41, further comprising a TAF-1 transactivation domain.

43. The molecular switch of claim 32 or 33, wherein said switch is tissue specific.

44. The molecular switch of claim 43, wherein the tissue specificity is determined by adding a transactivation domain which is specific to a given tissue.

45. The molecular switch of claim 43, wherein the tissue specificity is determined by the ligand which binds to the modified steroid hormone receptor.

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46. A method for regulating expression of a nucleic acid cassette in gene therapy comprising the step of attaching the molecular switch of claim 32 or 33, to a nucleic acid cassette to form a nucleic acid cassette/molecular switch complex for use in the gene therapy.

47. The method of claim 46 further comprising the step of administering a pharmacological dose of the nucleic acid cassette/molecular switch complex to an animal or human to be treated.

48. The method of claim 47, wherein said molecular switch is turned on or off by dosing the animal or human with a pharmacological dose of a ligand which binds to the modified ligand binding site.

49. A composition of matter comprising a molecular switch linked to a nucleic acid cassette, wherein said cassette/molecular switch complex is positionally and sequentially oriented in a vector such that the nucleic acid in the cassette can be transcribed and when necessary translated in a target cell.

50. A method for regulating nucleic acid cassette expression in gene therapy comprising the steps of:

forming a nucleic acid cassette/molecular switch complex by linking a molecular switch to a nucleic acid cassette in positional relationship such that the expression of the nucleic acid sequence in the nucleic acid cassette is capable of being up-regulated or down-regulated by the molecular switch;

inserting the nucleic acid cassette/molecular switch complex into a cell to form a transformed cell; and

inserting a pharmacological dose of the transformed cell into a human or animal for gene therapy.

51. The method of claim 50 for treating senile dementia or Parkinson's disease, wherein the nucleic acid cassette contains the nucleic acid sequence coding for a protein selected from the group consisting of a hormone, a neurotransmitter and a growth factor; and the transformed cell is a brain cell.

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52. The method of claim 51, further comprising the step of encapsulating the brain cell containing the nucleic acid cassette/molecular switch complex in a permeable structure, said permeable structure capable of allowing the passage of activators of the molecular switch and protein translated from the nucleic acid sequence but preventing passage of attack cells.

53. The method of claim 51 or 52, wherein the molecular switch is comprised of a progesterone receptor with the native ligand binding domain replaced with modified ligand binding domain which binds anti-progesterone.

54. The method of claim 50, 51 or 52 wherein the molecular switch is comprised of a progesterone receptor with the native DNA binding domain replaced with GAL-4 DNA binding domain.

55. The method of claim 50, 51 or 52, wherein the nucleic acid sequence is transcribed to produce a protein after the animal or human is given a pharmacological dose of an anti-progesterone.

56. The method of claim 55, wherein the amount of protein produced in the transformed cell is proportional to the dose of anti-progesterone.

57. The method of claim 53, wherein the molecular switch and a nucleic acid cassette are on separate plasmids and are co-injected into a target cell.

58. The molecular switch of claim 43, further comprising the addition of a tissue-specific cis-element to the target gene.

59. A molecular switch comprised of:

a VP-16 transcription region attached to a modified steroid hormone receptor, said receptor including a GAL-4 DNA binding domain and a modified ligand binding domain.

60. A molecular switch comprised of:

a TAF-1 transcription region attached to a modified steroid hormone receptor, said receptor including a GAL-4 DNA binding domain and a modified ligand binding domain.



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61. The molecular switch of claim 59 or 60, wherein the ligand binding domain binds a compound selected from the group consisting of 5-alpha-pregnane-3,2-dione; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -propinyl-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadiene-3-one; 11 $\beta$ -(4-acetylphenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(1-propinyl)-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one; (7 $\beta$ ,11 $\beta$ ,17 $\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2'(3'H)-furan]-3-one; (11 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-4',5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one.

62. A molecular switch for regulating expression of a nucleic acid cassette in a transgenic animal, comprising:

a modified steroid receptor, said receptor including a natural steroid receptor DNA binding domain linked to a modified ligand binding domain,

63. The molecular switch of claim 62, wherein the natural steroid receptor DNA binding domain has been replaced with a non-native or modified DNA binding domain.

64. The molecular switch of claim 62 or 63, wherein the ligand binding domain is modified to bind a compound selected from the group consisting of non-natural ligands, anti-hormones and non-native ligands.

65. The molecular switch of claim 62 or 63, wherein the ligand binding domain binds a compound selected from the group consisting of 5-alpha-pregnane-3,2-dione; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -propinyl-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadiene-3-one; 11 $\beta$ -(4-acetylphenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(1-propinyl)-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one; (7 $\beta$ ,11 $\beta$ ,17 $\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ster-4,9-diene-17,2'(3'H)-furan]-3-one; (11 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-4',5'-

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dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one.

66. The molecular switch of claim 63, wherein the modified steroid receptor is a receptor with the natural DNA binding domain replaced with a DNA binding domain selected from the group consisting of GAL-4 DNA, virus DNA binding site, insect DNA binding site and a non-mammalian DNA binding site.

67. The molecular switch of claim 62 or 63, further comprising linking a transactivation domain selected from the group consisting of VP-16, TAF-1, TAF-2, TAU-1 and TAU-2 to the modified steroid receptor.

68. The molecular switch of claim 62 or 63, wherein said switch is tissue specific.

69. The molecular switch of claim 68, wherein the tissue specificity is determined by adding a transactivation domain which is specific to a given tissue.

70. The molecular switch of claim 68, wherein the tissue specificity is determined by the ligand which binds to the modified steroid hormone receptor.

71. A method for regulating expression of a nucleic acid cassette in a transgenic animal comprising the step of attaching the molecular switch of claim 62 or 63, to a nucleic acid cassette to form a nucleic acid cassette/molecular switch complex for use in the transgenic animal.

72. The method of claim 71 further comprising the step of administering a pharmacological dose of the nucleic acid cassette/molecular switch complex to the transgenic animal.

73. The method of claim 72, wherein said molecular switch is turned on or off by dosing the transgenic animal with a pharmacological dose of a ligand which binds to the modified ligand binding site.

74. A composition of matter comprising a molecular switch linked to a nucleic acid cassette, wherein said cassette/molecular switch complex is positionally and sequentially oriented in a vector such that the nucleic

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acid in the cassette can be transcribed and when necessary translated in a target cell.

75. A method for regulating nucleic acid cassette expression in a transgenic animal comprising the steps of:

forming a nucleic acid cassette/molecular switch complex by linking a molecular switch to a nucleic acid cassette in positional relationship such that the expression of the nucleic acid sequence in the nucleic acid cassette is capable of being up-regulated or down-regulated by the molecular switch;

inserting the nucleic acid cassette/molecular switch complex into a cell to form a transformed cell; and

inserting a pharmacological dose of the transformed cell into the transgenic animal.

76. The method of claim 75, wherein the molecular switch is comprised of a progesterone receptor with the native DNA binding domain replaced with GAL-4 DNA binding domain.

77. The method of claim 75, wherein the nucleic acid sequence is transcribed to produce a protein after the transgenic animal is given a pharmacological dose of an anti-progesterone.

78. The method of claim 77, wherein the amount of protein produced in the transformed cell is proportional to the dose of anti-progesterone.

79. The method of claim 75, wherein the molecular switch and a nucleic acid cassette are on separate plasmids and are co-injected into a target cell.

80. The molecular switch of claim 68, further comprising the addition of a tissue-specific cis-element to the target gene.

81. The molecular switch of claim 68, further comprising the addition of progesterone responsive elements into a promoter of the nucleic acid cassette.

82. A molecular switch for regulating expression of a nucleic acid cassette in a plant, comprising:

a modified steroid receptor, said receptor including a natural steroid receptor DNA binding domain linked to a modified ligand binding domain,

83. The molecular switch of claim 82, wherein the natural steroid receptor DNA binding domain has been replaced with a non-native or modified DNA binding domain.

84. The molecular switch of claim 82 or 83, wherein the ligand binding domain is modified to bind a compound selected from the group consisting of non-natural ligands, anti-hormones and non-native ligands.

85. The molecular switch of claim 82 or 83, wherein the ligand binding domain binds a compound selected from the group consisting of 5- $\alpha$ -pregnane-3,2-dione; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -propinyl-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadiene-3-one; 11 $\beta$ -(4-acetylphenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(1-propinyl)-4,9-estradiene-3-one; 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(3-hydroxy-1(Z)-propenyl)-estra-4,9-diene-3-one; (7 $\beta$ ,11 $\beta$ ,17 $\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro[ester-4,9-diene-17,2'(3'H)-furan]-3-one; (11 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-4',5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4,9-diene-17,2'(3'H)-furan]-3-one.

86. The molecular switch of claim 83, wherein the modified steroid receptor is a receptor with the natural DNA binding domain replaced with a DNA binding domain selected from the group consisting of GAL-4 DNA, virus DNA binding site, insect DNA binding site and a non-mammalian DNA binding site.

87. The molecular switch of claim 82 or 83, further comprising linking a transactivation domain selected from the group consisting of VP-16, TAF-1, TAF-2, TAU-1 and TAU-2 to the modified steroid receptor.

88. The molecular switch of claim 82 or 83, wherein said switch is tissue specific.

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89. The molecular switch of claim 88, wherein the tissue specificity is determined by adding a transactivation domain which is specific to a given tissue.

90. The molecular switch of claim 88, wherein the tissue specificity is determined by the ligand which binds to the modified steroid hormone receptor.

91. A method for regulating expression of a nucleic acid cassette in a plant comprising the step of attaching the molecular switch of claim 82 or 83, to a nucleic acid cassette to form a nucleic acid cassette/molecular switch complex for use in the plant.

92. The method of claim 91 further comprising the step of administering a pharmacological dose of the nucleic acid cassette/molecular switch complex to the plant.

93. The method of claim 92, wherein said molecular switch is turned on or off by dosing the plant with a pharmacological dose of a ligand which binds to the modified ligand binding site.

94. A composition of matter comprising a molecular switch linked to a nucleic acid cassette, wherein said cassette/molecular switch complex is positionally and sequentially oriented in a vector such that the nucleic acid in the cassette can be transcribed and when necessary translated in a target cell.

95. A method for regulating nucleic acid cassette expression in a plant comprising the steps of:

forming a nucleic acid cassette/molecular switch complex by linking a molecular switch to a nucleic acid cassette in positional relationship such that the expression of the nucleic acid sequence in the nucleic acid cassette is capable of being up-regulated or down-regulated by the molecular switch;

inserting the nucleic acid cassette/molecular switch complex into a cell to form a transformed cell; and

inserting a pharmacological dose of the transformed cell into the plant.

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96. The method of claim 95, wherein the molecular switch is comprised of a progesterone receptor with the native DNA binding domain replaced with GAL-4 DNA binding domain.

97. The method of claim 95, wherein the nucleic acid sequence is transcribed to produce a protein after the plant is given a pharmacological dose of an anti-progesterone.

98. The method of claim 97, wherein the amount of protein produced in the transformed cell is proportional to the dose of anti-progesterone.

99. The method of claim 95, wherein the molecular switch and a nucleic acid cassette are on separate plasmids and are co-injected into a target cell.

100. The molecular switch of claim 68, further comprising the addition of progesterone responsive elements into a promoter of the nucleic acid cassette.

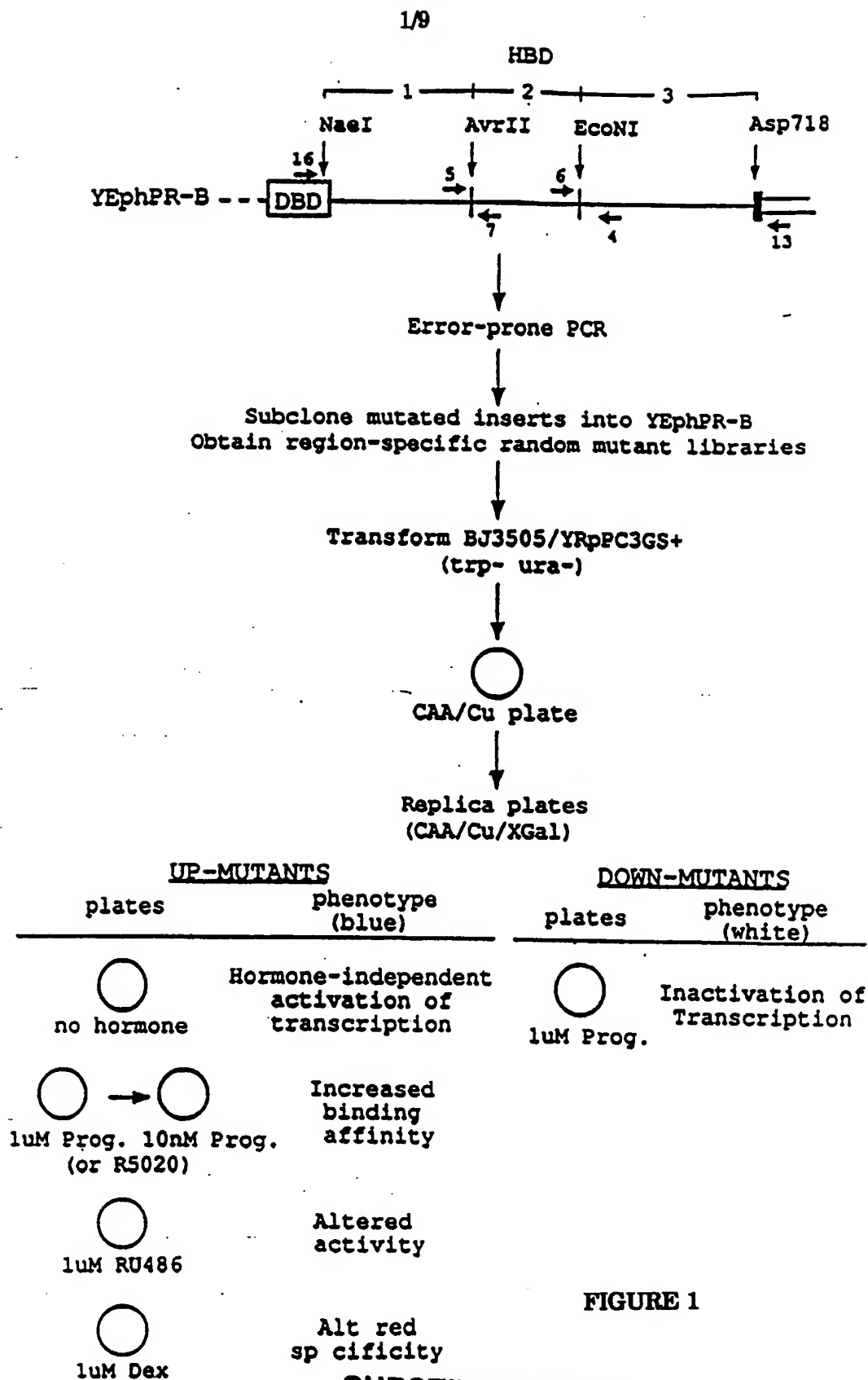


FIGURE 1

SUBSTITUTE SHEET

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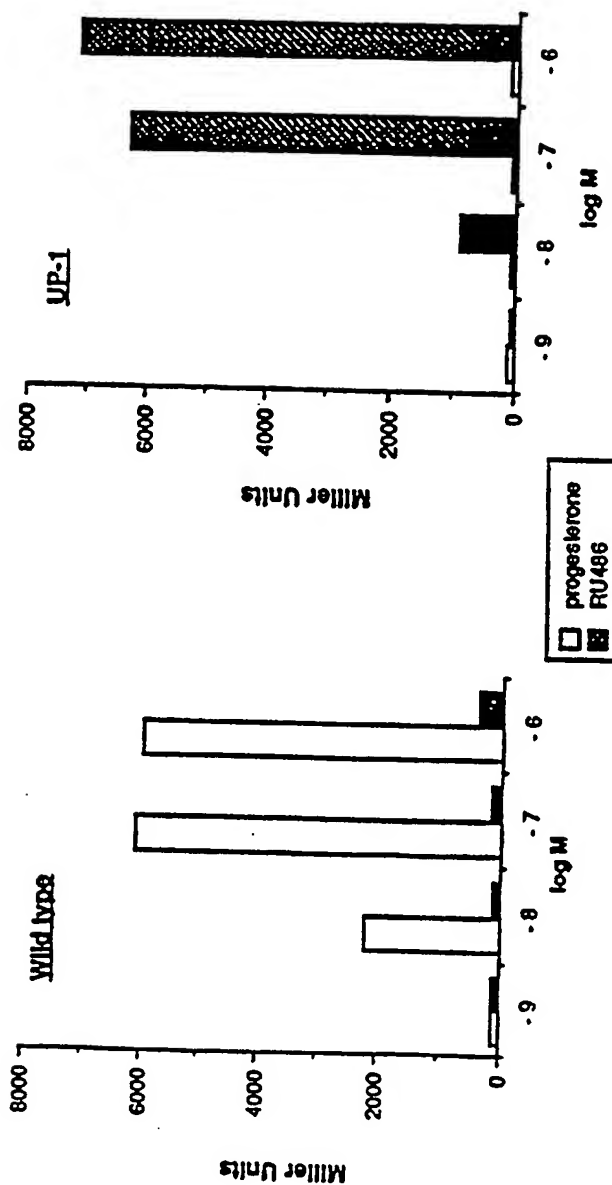


FIGURE 2B

FIGURE 2A



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DNA sequence:

WT                   2636  
 ..AAC TTG CAT GAT CTT GTC AAA CAA CTT CAT CTG TAC TGC TTG..  
 UP-1..AAT TGC ATG ATC TTG TCA AAC AAC TTC ATC TGT ACT GCT TGA

Protein sequence:

WT                   879                   891  
 ..Asn Leu His Asp Leu Val Lys Gln Leu His Leu Tyr Cys Leu..  
 UP-1..Asn Cys Met Ile Leu Ser Asn Asn Phe Ile Cys Thr Ala

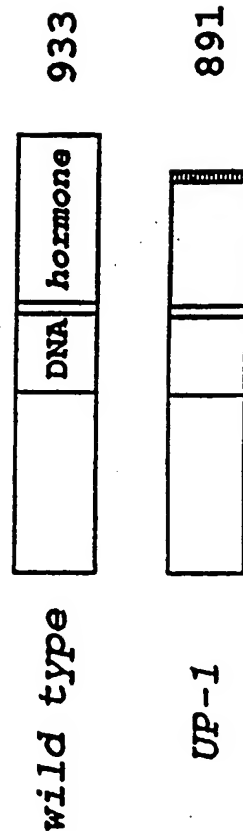


FIGURE 2C

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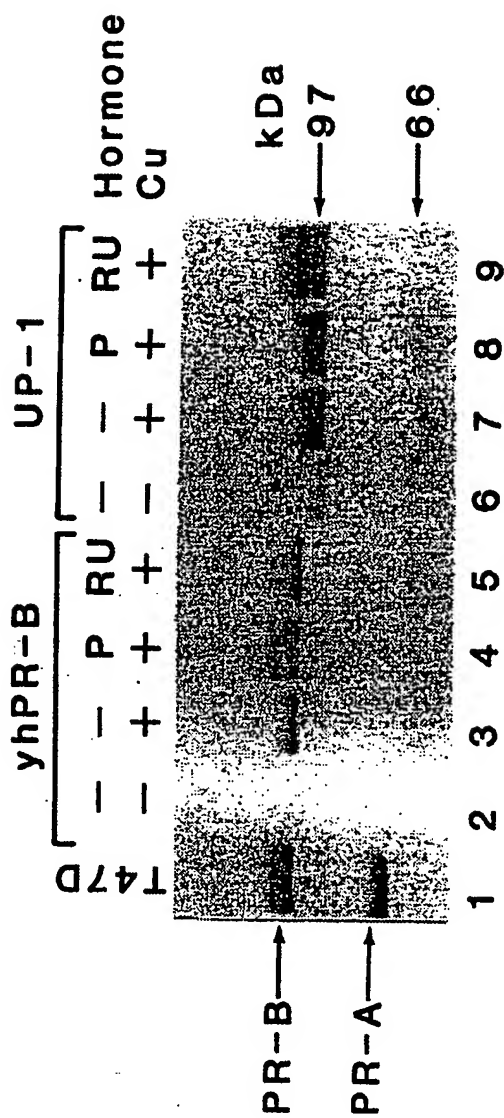


FIGURE 3

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hPR Constructs	Transcriptional Activity (Miller Units)			Specific Binding (nM)		
	-	P	RU	P	RU	RU
YEphPR-B (933)		86	6200	586	1.0	1.3
UP-1		286	466	8050	0.02	1.6
YEphPR-B879		166	242	5900	0.04	1.8
YEphPR-B891		243	226	6175	0.03	1.6

FIGURE 4

SUBSTITUTE SHEET

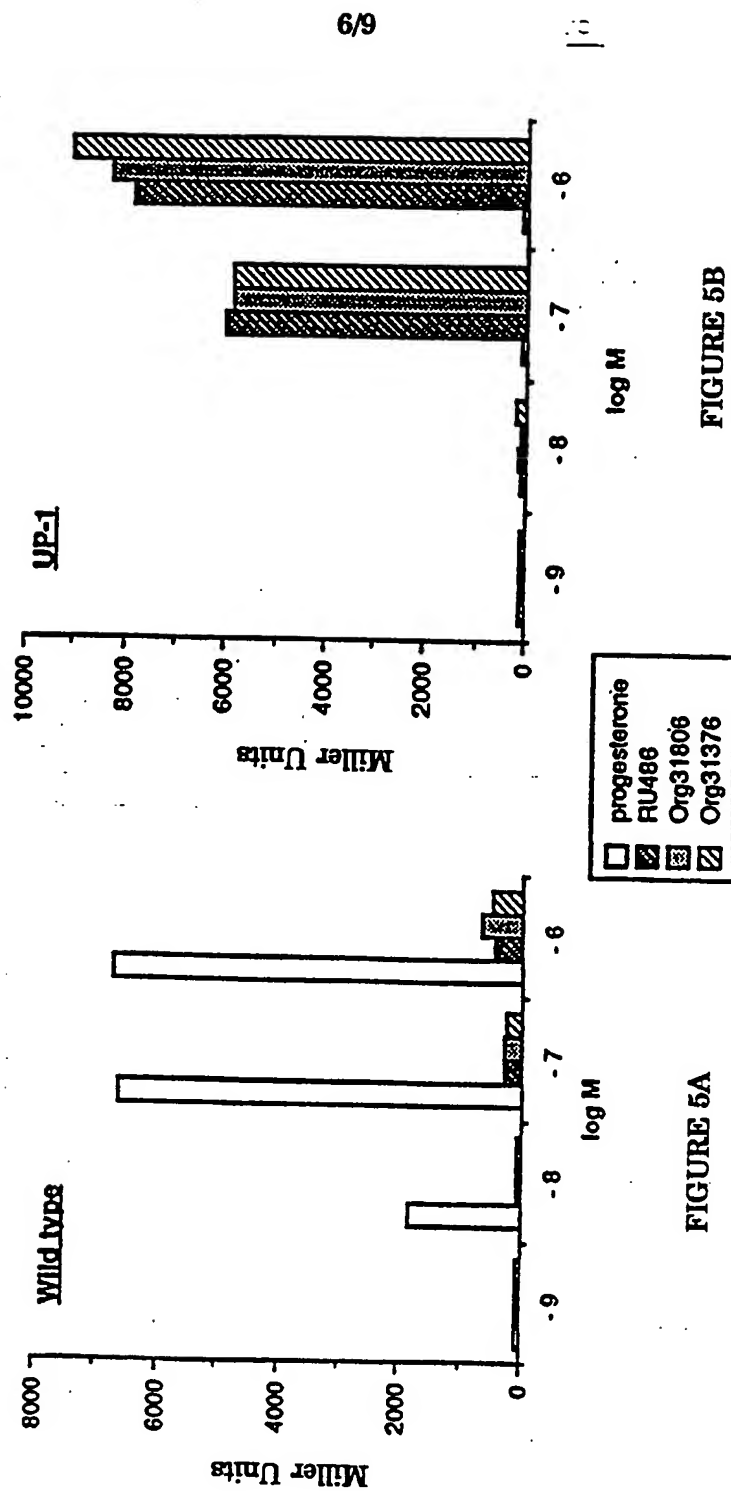


FIGURE 5B

FIGURE 5A

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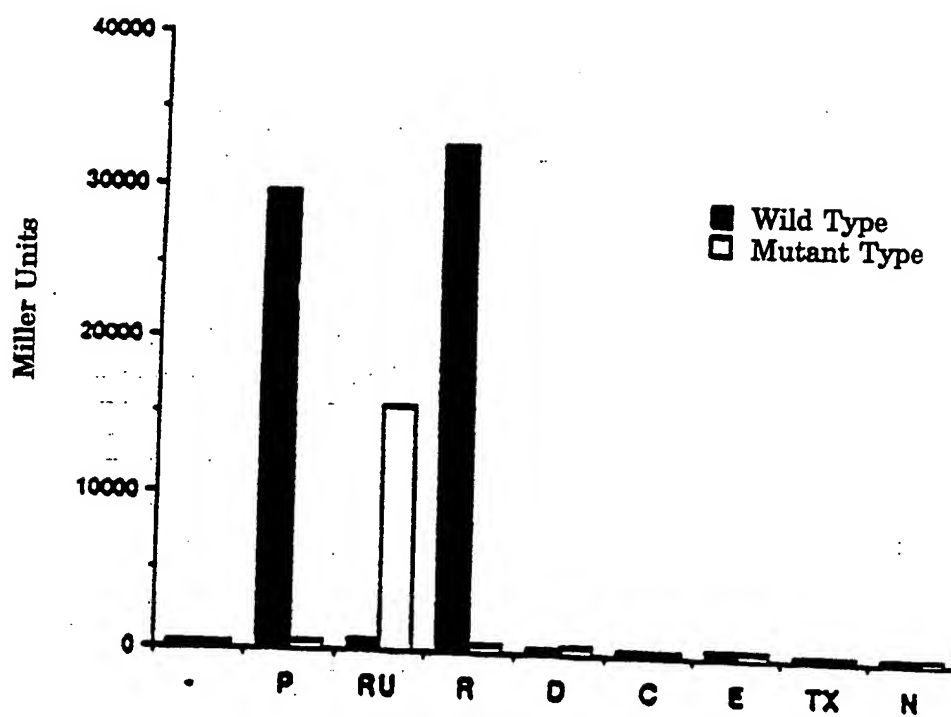


FIGURE 5C

SUBSTITUTE SHEET

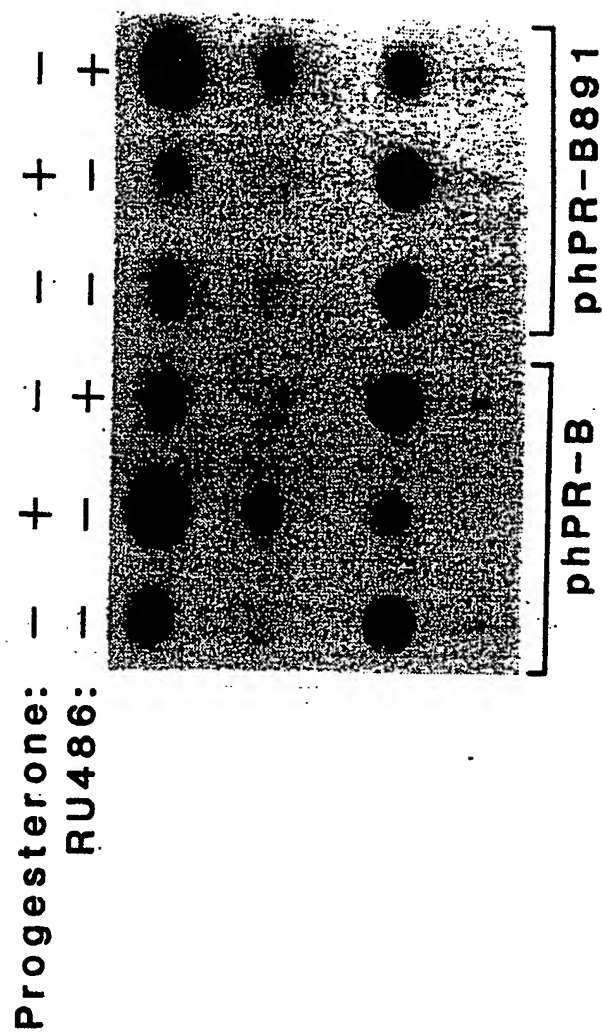


FIGURE 6A

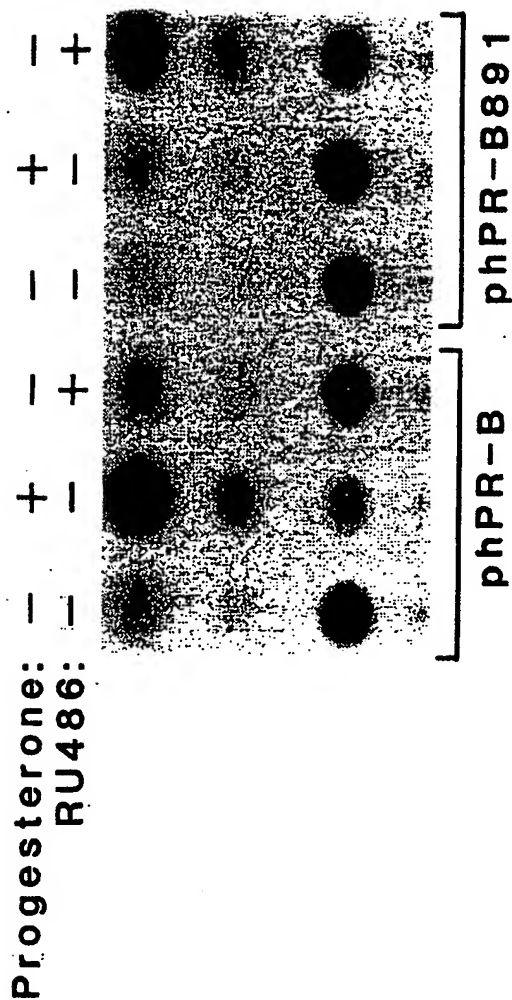


FIGURE 6B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04399

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 264, No. 7, issued 05 March 1989, A. D. W. Dobson et al., "Mutational Analysis of the Chicken Progesterone Receptor", pages 4207-4211, especially page 4208.	1-100
Y	Science, Volume 255, issued 10 January 1992, B. Benhamou et al., "A Single Amino Acid That Determines the Sensitivity of Progesterone Receptors to RU486", pages 206-209, especially pages 207-208.	1, 2, 7-100
Y	Nature, Volume 335, issued 20 October 1988, M. Ptashne, "How Eukaryotic Transcriptional Activators Work", pages 683-689, especially page 685.	13, 37, 54, 58, 66, 76, 86, 96

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 JULY 1993

Date of mailing of the international search report

02 AUG 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04399

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The EMBO Journal, Volume 9, No. 12, issued 1990, M.-E. Meyer et al., "Agonistic and Antagonistic Activities of RU486 on the Functions of the Human Progesterone Receptor", pages 3923-3932, especially pages 3923-3926.	38-40, 42, 60, 67, 87
Y	Molecular Endocrinology, Volume 5, No. 11, issued 1991, K. Christensen et al., "Characterization and Functional Properties of the A and B Forms of the Human Progesterone Receptors Synthesized in a Baculovirus System"; pages 1755-1770, especially pages 1767-1768.	15
Y	Proceedings of the National Academy of Science, Volume 84, issued November 1987, C.-Y. Wang et al., "pH-Sensitive Immunoliposomes Mediate Target-Cell-Specific Delivery and Controlled Expression of a Foreign Gene in Mouse", pages 7851-7855, especially page 7854.	32-100
Y	Contributions to Cellular and Molecular Endocrinology - Published Proceedings of the 10th Annual University of California, Riverside-Nichols Institute Symposium on Cellular and Molecular Endocrinology, issued 1988, R. M. Evans, "The Superfamily of Ligand-Responsive Transcription Factors", pages 132-142, especially page 132.	41
Y	US, A, 4,892,538 (Aebischer et al.) 09 January 1990, column 1, line 23 to column 3, line 52.	51-53

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04399

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (5):

C07K 13/00; C12N 5/00, 15/00; C07H 15/12, 17/00; G01N 33/566; A61K 48/00

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

424/422, 93B; 435/7.1, 69.4, 172.3, 240.1, 240.22, 320.1; 436/501; 514/44; 530/350; 536/23.1, 23.5

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

424/422, 93B; 435/7.1, 69.4, 172.3, 240.1, 240.22, 320.1; 436/501; 514/44; 530/350; 536/23.1, 23.5; 935/10, 11, 34, 36, 37, 66, 69, 70, 71

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS,

search terms: antiprogesterin, steroid, receptor, agonist, antagonist, RU486, assay, transcription, gene(w)therapy, molecular(w)switch, progesterone, parkinson?, brain, transplant, implant, permeable, chimera?, chimera?, cells, graft, engraft